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DOI: https://doi.org/10.1111/tra.12387

Principles of Virus Uncoating: Cues and the Snooker Ball

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Keywords:

virus structure; signalling; endocytosis; membrane fusion; penetration; low pH; cytoskeleton; nuclear import; nuclear pore complex; molecular motor

Running title: Virus entry and uncoating

Synopsis:

The architecture of a virus particle allows timely release of the viral genome in a host cell during entry. This critical step is known as viral uncoating. It is regulated by cues from receptors, enzymes and chemicals, and facilitated by factors that do not contact the virion directly. This review covers a wide range of cellular processes that enhance viral uncoating. The underlying mechanisms provide deep insights into cell biological and immunological processes of virus-host interactions and infections.

Abstract

Viruses are spherical or complex shaped carriers of proteins, nucleic acids and sometimes lipids and sugars. They are metastable and poised for structural changes. These features allow viruses to communicate with host cells during entry, and to release the viral genome, a process known as uncoating. Studies have shown that hundreds of host factors directly or indirectly support this process. The cell provides molecules that promote stepwise virus uncoating, and direct the virus to the site of replication. It acts akin to a snooker player who delivers accurate and timely shots (*cues*) to the ball (*virus*) to score. The viruses, on the other hand, trick (snooker) the host, hijack its homeostasis systems, and dampen innate immune responses directed against danger signals. In this review, we discuss how cellular cues, facilitators, and built-in viral mechanisms promote uncoating. Cues come from receptors, enzymes, and chemicals that act directly on the virus particle to alter its structure, trafficking and infectivity. Facilitators are defined as host proteins that are involved in processes which indirectly enhance entry or uncoating. Unraveling the mechanisms of virus uncoating will continue to enhance understanding of cell functions, and help counteracting infections with chemicals and vaccines.

How cellular cues and facilitators shape the viral uncoating program

Virus entry is the process by which the genome of a virus particle is delivered to the replication site, which can be in the cytosol, on cytoplasmic membranes or in the nucleus. The viral uncoating program is encoded in the viral genome together with the blueprint for the production of progeny viruses. The majority of viruses link their uncoating program to the endocytic machinery (for summary, see **Figure 1**) (1). The details of uncoating are highly variable depending on the nature of the virus and the cell. However, the profile of events is similar for most viruses. It involves a stepwise process with a final step, the release of the genome from a protective, confining capsid structure (2, 3). The final step enables transcription and replication, or in the case of DNA and RNA retroviruses the stable maintenance of the viral genome in the host nucleus. As a rule, complete uncoating occurs once the capsid has reached its final destination (4). The steps of uncoating are regulated by cellular cues, which directly act on the virus. Here we have categorized cellular factors exerting cues to promote entry and uncoating of the incoming particle. The three major cues come from host receptors, enzymes and small chemicals including ions.

Receptor cues come from plasma membrane associated molecules (proteins, sugars, lipids). They bind the virus to a cell, and actively promote virus endocytosis. They mediate conformational changes in the virion (a virus particle outside the cell) or the virus, and promote the formation of microdomains that trigger signaling pathways and enable the infection process (1, 4-7). Signaling plays a critical role in virus entry. Receptors often follow the virus into the cell during endocytosis, as shown with dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and bunyavirus (8). Intracellular receptors found in the endocytic pathway can also play an important role in viral fusion and escape into the cytosol. Examples are Niemann-Pick C1 (NPC1) for Ebola virus and lysosome associated membrane protein 1 (LAMP1) for Lassa virus (9, 10). A receptor cue and actomyosin work together in virion surfing at the extracellular part of the plasma membrane (11-15).

Enzymatic cues include oxido-reduction, quality control machineries such as the ubiquitin proteasome system (UPS), endoplasmic reticulum (ER)-associated protein degradation (ERAD) and disaggregation. A good example is simian virus 40 (SV40), a polyomavirus whose uncoating process in the ER has been studied in detail (16-23) (see also chapter 'Uncoating cues and facilitators'). Further to this, protease digestion can activate viral spike proteins that facilitate virus fusion with host membranes and escape of many viruses from endosomes (24). Enzymatic activity can also result in generation of mechanical/physical forces involving cytoskeletal motors (dynein, kinesin, myosin), the cytoskeleton (tubulin, actin filaments), and the nuclear pore complex (NPC). Such forces can alter the physical properties of the capsid and promote uncoating and genome release (11, 25, 26).

Chemical cues such as low pH, and changes in other ion concentrations are, for example, spatiotemporally regulated during endosome maturation (27, 28). They trigger conformational changes in the viral envelope, the coat or the nucleic acid-protein core of incoming viruses (29, 30). Changes in chemical properties between different cellular compartments, such as from ER to cytosol (16), or endosome to cytosol, can promote further steps of uncoating.

Facilitators are host factors (proteins, sugars, lipids, ions) that enhance viral uncoating, but do so in an indirect manner compared to the cues. According to current knowledge, facilitators do not directly bind to the virus. Examples include cell signaling molecules, such as Ca^{2+} transients in the cytosol, endosome maturation factors, or intracellular transport factors, such as actin and microtubule filaments.

A summary of cellular cues and processes implicated in virus uncoating is shown in **Figure 2**.

Viral strategies that promote uncoating

Viruses have built-in mechanisms that respond to cellular cues and facilitators, in order to promote entry and uncoating. For example, by containing phosphatidylserine (PS) within their membranes viruses can effectively mimic apoptotic cells, thereby subverting apoptotic clearance mechanisms to facilitate entry or infection (31). Below we list some of the strategies used by viruses.

a) Low pH-activated viral fusion. Virus-cell fusion is the means by which all enveloped viruses, including HIV, influenza and Ebola virus enter cells. It requires bringing two separate membrane bilayers into intimate contact and then merging them into one. It is executed by one or more viral surface glycoproteins. The sole cue so far known to trigger fusion of orthomyxo-, rhabdo-, alpha-, flavi-, bunya-, and arenaviruses is low pH (32). A recently discovered family of cellular factors with anti-viral activity are interferon inducible transmembrane (IFITM) proteins. They have the ability to inhibit viral entry, possibly by modulating the fluidity of cellular membranes and blocking fusion (33).

b) Low pH-activated viral protease and glycosidase. Some viruses activate proteolytic activity in response to an acidic environment. Adeno-associated virus (AAV) is capable of autolytic cleavage at multiple sites within the capsid, which is induced at pH 5.5 (34). This cleavage may contribute to the escape of AAV from acidic endosomes during entry. The (NA) of H5N1 influenza A virus was shown to have high catalytic activity at low pH, and to cleave glycosylated LAMPs. The main activity of NA is to release newly assembled virus particles from the cell surface (35). NA inhibitors can also reduce early stages of infection, and it is possible that NA activity in late endosomes is required for optimal virus entry (36).

c) Capsid softening and internal pressure. Using atomic force microscopy (AFM) at virion resolution, it was found that influenza virus particles soften following acidification of the viral core. This is due to acid-induced conformational changes of the capsid independent of the viral glycoproteins (37-39). Assembled adenovirus particles contain an estimated internal pressure of 30 atmospheres, which is thought to assist in the stepwise virus disassembly process, starting at the physically weakest spot of the virus, the five-fold symmetrical vertex (2, 11, 40-44). Chemical and mechanical stability have been known to correlate in bacteriophage T7 (45), and internal pressure with capsid stiffness in phage phi29 (46). The resistance of the adenovirus vertex depends on virus maturation, which is mediated by the adenovirus cysteine protease AVP (47, 48), and also on innate factors against the virus, such as defensins which bind to and stabilize the vertex region (49). Pressure within the virus particle is built up by electrostatic repulsion between the negatively charged dsDNA strands, DNA bending and entropic components, and is thought to weaken the pentons (50). It eventually facilitates genome ejection for DNA translocation into the nucleus, a strategy acquired by bacteriophage and certain eukaryotic viruses such as herpesviruses (46, 51).

d) Directional genome uncoating. Human rhinoviruses (HRVs) are the major cause of the common cold. The uncoating process of the minor group virus HRV2 begins with low density lipoprotein receptor (LDLR) binding, and clathrin-dependent and -independent endocytosis (52, 53). Conversion of the capsid to subviral particles is induced by low pH in late endosomes, and VP1 and VP4 insert into the endosomal lumen creating an ion-conducting pore (30, 54, 55). The 3'-end of the linear RNA genome exits from the capsid first, suggesting that the RNA adopts a defined conformation inside the viral capsid (56).

e) The assembly-disassembly paradox. How can a virus be assembled in an infected cell and fall apart (disassemble) during entry into an uninfected cell? One possibility is that virus capsid is assembled as a stable structure in an infected cell and rendered metastable, for example by limited proteolysis, such that it can receive cues from the host (3). A well studied example here is adenovirus (48). A second possibility is that the virus particle itself is unchanged during assembly and egress, but that the infected and uninfected cells are different. An uncoating factor may be absent (inactive) during assembly, but present (active) during viral entry. Semliki Forest virus (SFV) (see Box 1) and influenza virus uses this strategy (25, 57, 58). During assembly of influenza virus, proton flux through the viral M2 ion channel, which is present in the Golgi membrane, neutralizes the acidic pH of the trans-Golgi network (TGN) and thereby prevents activation of the newly synthesized hemagglutinin (HA) to its fusogenic form (59-62). A third possibility is that the virus is unchanged but assembly and uncoating are spatially separated (3, 63). Polyomaviruses, for example, are uncoated in the ER, but assembled inside the nucleus (18, 64). Influenza viral ribonucleoprotein (vRNPs) are prevented from re-import after replication, or following microinjection into infected cells due to vRNP binding to newly expressed viral matrix protein (M1) (65, 66). Replicated adenovirus DNA cannot undergo inter-nuclear spreading upon fusion of an infected cell with uninfected neighboring cells (67). To be infectious, progeny viruses must be released and go through an uncoating program during entry into a new cell.

As we described above, viruses have evolved to navigate the networks of host genes, proteins, lipids and RNAs, as well as metabolic and catabolic

pathways. This allows viruses to couple their stepwise disassembly with the entry process into cells, leading them through chemically distinct cellular environments.

Uncoating at the nuclear pore

Many viruses replicate in the cytoplasm. Others travel into the nucleus for replication (68). For such viruses nuclear entry is a limiting step in infection. The vertebrate nuclear pore complex (NPC) has an estimated molecular mass of 125 MDa and is composed of 80-100 different proteins called nucleoporins (Nups) (69-71). The NPC has barrier and transport functions, with kinetic cargo size restriction of about 39 nm for receptor-mediated transport and solutes of about 40 kDa (71, 72). Cargo docking sites surround the pore on the cytoplasmic side, and the NPC structure constricts to form a dynamic basket on the nuclear side (73). The NPC is a major bottleneck for viruses to overcome during cell entry because it provides the only continuous aqueous connection between the cytoplasm and the nucleus (74). Nuclear entry and uncoating are often concomitant.

a) NPC docking and genome release. Large virus capsids, such as those of herpesvirus (125 nm in diameter) and adenovirus (70-90 nm), are too large to enter the nucleus through the NPC. Instead, they follow a pathway of stepwise uncoating and weakening of the capsid to release their linear, double stranded (ds) DNA into the nucleus.

Herpes simplex virus-1 (HSV-1) fuses at the plasma membrane, although endosomal fusion has also been reported (75). Capsids shed some outer tegument proteins into the cytosol. Some of these proteins, such as the major tegument protein VP16 (a potent transcription factor) are imported into the nucleus to enhance viral immediate early gene expression (76-78). The capsids and tightly bound inner tegument proteins bind to dynein and kinesin motors that regulate retrograde transport on microtubules toward the nucleus (76, 79-81). After docking to the NPC via Nup358 or Nup214, a single vertex of the capsid is opened, and viral DNA is released into the nucleus possibly by high internal capsid pressure (82-87). This model is analogous to DNA ejection by bacteriophages into bacterial cells (88).

Adenovirus capsids, following endocytosis and endosomal escape, are transported along microtubules towards the nucleus, where they dock at the NPC via Nup214 and are disassembled by the outward pulling force generated by kinesin-1 and microtubules (26, 89-91). After the capsid is disassembled, the viral genome translocates into the nucleus using nuclear import receptors and histone H1 (90-96). However, some viral DNA fail to be properly delivered into or retained within the nucleus (95, 97). This DNA misdelivery may give rise to inflammatory host responses which is a widespread feature of human adenovirus infections (98).

b) Small viruses. Viruses with capsids smaller than about 40-50 nm, such as hepatitis B virus (HBV), parvoviruses and some polyomaviruses, can enter through the pores in either an intact form or as a subviral particle. HBV enters the nucleus to generate a covalently closed circular viral DNA genome (cccDNA) and to transcribe this genome. During the import process, immature capsids are initially trapped within the NPC via Nup153, and then undergo a maturation process and disassembly, which releases viral DNA and attached viral DNA polymerase to the nucleus by an nuclear localization signal (NLS)-dependent process (99, 100). Nuclear cccDNA is maintained in infected hepatocytes, and used for reduplication and assembly of progeny virus. The high persistence of cccDNA makes pharmacological treatment of chronically HBV infected individuals difficult (101, 102). Parvoviruses are thought to enter the nucleus as intact particles, or by transiently disrupting nuclear membranes, followed by disassembly in the nucleus, albeit mechanisms are unknown (103, 104).

c) Rod-shaped genomes. Several negative-stranded RNA viruses such as influenza, Thogoto, and Borna disease viruses replicate their RNAs in the nucleus (105). Influenza circumvents the size limit of the NPC by encoding its genetic information on eight separate, rod-like vRNPs that are thin enough in diameter to enter through the pore. They resemble a twisted rod (10-15nm in width and 30-120nm in length) that is folded back and coiled on itself (106, 107). Studies using amantadine have shown that acidification of the viral core in endosomes via the viral M2 channel is essential for the dissociation of incoming nucleocapsids in the cytosol following fusion (108, 109). After capsid uncoating, the vRNPs are released into the cytosol. Nucleoprotein (NP), the main component of vRNPs, contains NLSs necessary for nuclear import (110-115). Progeny vRNPs are exported to the cytosol after binding to M1 and nuclear export protein (NEP), and are prevented from re-import into the nucleus (116-119).

d) Uncoating factors and immune evasion. Human immunodeficiency virus type 1 (HIV-1) uncoating is controlled by host factors. Following fusion at the plasma membrane, cyclophilin A or TRIM5α destabilize the capsid. Transportin 3 (TNPO3), Nup358, cleavage and polyadenylation specificity factor subunit 6 (CPSF6), dynein, kinesin-1 and components of the NPC regulate nuclear transport of the capsid and import of reverse transcribed DNA (120-128). In primary human macrophages, it was shown that recruitment of

cyclophilin A or CPSF6 to capsid protein (CA) prevents premature DNA synthesis, innate recognition, and interferon (IFN)-dependent restriction of HIV-1 (129). In addition, cytoplasmic pools of Nups may control uncoating by binding to the capsid, or mediate capsid or core docking to the NPC (130). TNPO3, a member of the importin β family, might play a role in displacing CA and tRNA from the preintegration complex in the nucleus, and thereby facilitate integration of the viral genome into host chromatin (127, 131).

e) Actin nucleation mediates nuclear targeting. During entry viruses use microtubule-based mechanisms to traffic through the cytoplasm to the nucleus for replication (68). Baculoviruses are an exception, and after fusion at the plasma membrane the nucleocapsids move on actin tails through the cytoplasm in random directions (132). When they collide with the nucleus, they may be proximal to an NPC and dock to it for a few minutes. Thereafter the nucleocapsid (diameter 30-60nm) is thought to squeeze through the pore, as suggested by electron micrographs (133), followed by uncoating in the nucleus. Actin nucleation of baculovirus is mediated by Arp2/3 (134) and depolymerization of actin inhibits viral entry and infection kinetics (132).

f) Mitosis for nuclear access. Unlike influenza virus, HIV-1, herpes-, and adenoviruses, HPV entry into the nucleus apparently does not require functional NPC. HPV16 is the causative agent of cervical cancer (135). It has evolved a strategy contingent with cell tropism in mucosal epithelia, and the skin, involving basal keratinocytes, which can be infected upon wounding. It establishes persistent infection. After endocytosis, the virus travels from the endosome to the TGN and ER in a retromer- and γ-secretase dependent manner (136, 137). Furthermore, access of the subviral DNA/L2 complex to the nucleus depends on mitotic breakdown of the nuclear membrane (138, 139), or direct transfer from the ER into the nucleus during reassembly of the nuclear envelope (137). This correlates with the notion that papillomavirus exclusively infects basal stem cells that undergo cell division (140). Gamma-retroviruses must also wait for nuclear membrane breakdown during mitosis for nuclear delivery of preintegration complexes (141-143).

(Box1) Break shot – technologies that advance virus entry studies

Light microscopy, electron microscopy, and correlative microscopy. The imaging field has seen enormous progress in recent years, including wide field microscopy and total internal reflection microscopy (TIRF), atomic force microscopy (AFM), automated high-throughput imaging, lattice light-sheet microscopy, and super resolution microscopy (25, 95, 144, 145). Advance and dissemination of technology has revolutionized the field of virus entry. Imaging-based entry assays have been developed to quantify distinct step of virus entry (146, 147). Ethynyl-nucleotide labeling using EdA/U/C and CLICK chemistry enables temporal and spatial mapping of the virus genome of adeno, vaccinia, papilloma, and herpes virus (95, 148). The same technique was used to detect cellular DNA synthesis upregulation by paracrine signaling from a herpesvirus-infected cell to a remote, uninfected cell (149). Fluorescence in situ hybridization (FISH) has been used to detect incoming viral genomes and viral genome uncoating for coronavirus, adenovirus, rhinovirus or influenza virus (91, 150, 151). It is of note, however, that FISH is inherently non-quantitative, as the procedure tends to underestimate the amount of cytoplasmic viral genomes due to extraction in the denaturation process (91). This clearly favors bioorthogonal click-chemistry based approaches that have single molecule sensitivity (95, 152). Wide field fluorescence microscopy is increasingly used for automated analysis and quantification of virus spreading phenotypes in cell cultures (153, 154). For example, Plaque2.0 is a high-throughput software method yielding multi-parametric datasets of virus spreading in 2-D monolayers. It is compatible with immuno-cytochemistry and FISH. Proximity-dependent DNA ligation assay (PLA) has been used to detect incoming papillomavirus to the TGN and ER (136, 137, 155). Fluorescence correlation spectroscopy (FCS) was used to study directional exit of the rhinovirus genomic RNA from the capsid (56). At the ultrastructural level, the combination of light and electron microscopy using a correlative light electron microscopy approach provides a powerful tool for the study of dynamic intracellular membrane trafficking events, virus entry and replication with high sensitivity and spatial precision (156-159). Such technologies may, in the future, enable detection of particular virus entry steps with greater accuracy and resolution, and give deeper insight into cell-cell variability of infection events.

Reverse genetics. The advent of reverse genetics and molecular engineering of RNA viruses has transformed the field of virology by permitting study of targeted genetic changes in virus genomes (for examples see (160-162)). Pseudoviruses and virus like particles (VLPs) can be generated from cloned cDNAs, for example, allowing for reverse genetics and introduction of fluorescent probes (163-167). Beta-lactamase (BlaM) viral core chimeras, such as BlaM fused to HIV-1 viral protein R (vpr) can be used to detect virus uncoating with high sensitivity (168, 169).

Systems Virology. Systems-level analyses are potent hypothesis generators, and analyses of systems data, in combination with mathematical modelling, are used to generate comprehensive, integrated and predictive

models of biological systems and virus-host interactions (170). Over the last decade, genome-wide RNAi screens have provided novel leads to study virus-host interactions (171). For example, such screens found that the interferon inducible transmembrane (IFITM) proteins are involved in entry of H1N1 influenza, West Nile, and Dengue virus (172). A recently established trifunctional reagent for ligand derivatization, termed TRICEPS, can be used to identify potential viral receptors on the cell surface (173). The proteomics informed by transcriptomics (PIT) technique allows *in silico* derivation of proteomes from transcriptomes. This allows generation of viral and host protein databases for non-model species (174). Quantitative proteomics can provide essential information on posttranslational protein modifications and interactions with other proteins. Among recent progress are studies with hepatitis C and influenza viruses (175-178), identifying serum response factor binding protein 1 as a potential uncoating factor for hepatitis C (177). VirScan is a versatile, high-throughput method to comprehensively analyze antiviral antibodies displaying proteome-wide coverage of peptides from all human viruses, from a single drop of blood (179). Evidently the synergy between molecular biology, viral immunology and systems virology reveals novel insights into virus entry and infection biology.

(BOX2) SFV – a model virus to study entry, uncoating, and immune evasion. In 1980, Ari Helenius and coworkers visualized the binding of Semliki Forest Virus (SFV), a relatively simple enveloped virus to the cell surface of BHK-21 cells, virus internalization into coated vesicles, and accumulation in intracellular vacuoles. They found that penetration of SFV was triggered by low pH. The virus responded to this chemical cue by activating the fusion spike glycoproteins, which then mediated fusion of the viral envelope with the limiting membrane of the early endosome, followed by penetration of the viral capsid into the cytosol (180, 181). Within about a minute of reaching the cytoplasm the capsid was fully uncoated by the 28S large ribosomal subunit, leaving the viral RNA bound to the cytosolic surface of endosomes, where replication occurred (182, 183). Viruses that failed to escape from endosomes were delivered to lysosomes and eventually degraded (184). Infected cells became resistant to superinfection with SFV but not influenza virus. It is thought that the uncoating capacity of the ribosomes is inactivated during SFV replication precluding superinfection, and protecting progeny capsids from disassembly (57). Helenius and coworkers recently showed that immediately after uncoating the cellular RNA helicase Upf1 and nonsense-mediated mRNA decay (NMD) (185-187) restrict SFV replication by degrading incoming genomic (+)RNA (188). It is possible that alphavirus replicases compete with NMD to evade host restriction.

Uncoating cues and facilitators – many viruses, diverse mechanisms

Viruses make use of ubiquitous cellular processes to execute their uncoating program. These processes, ironically, often serve to maintain the cell in a healthy state. For example, accumulation of protein aggregates is a feature of cellular stress and aging in all organisms and associated with pathology. Protein disaggregation is central to the establishment of homeostasis and long term cell survival (189). An emerging feature of viral entry and uncoating is the mimicking of misfolded protein aggregates, so called 'waste proteins', in order to hijack cellular quality control processes that dispose of waste.

a) Aggresome processing. Influenza virus is an enveloped virus. It uses histone deacetylase 6 (HDAC6) for uncoating. HDAC6 plays a central role in regulating both the concentration and autophagic clearance of protein aggregates (25, 190-193). After binding to sialic acids at the cell surface, influenza virus is taken up by both clathrin-mediated endocytosis (CME) and macropinocytosis (194-197). Virus uptake is facilitated by receptor tyrosine kinase signaling that is mediated by epidermal growth factor (EGFR) (198). The viral core is primed for uncoating in endosomes by H^+ and K^+ influx through the M2 ion channel (199-203), followed by HA-mediated viral fusion at late endosomes which depends on low pH, CD81 and cathepsin W (204-206). The capsid then exposes unanchored ubiquitin chains, a hallmark of misfolded protein aggregates. These chains are likely generated by deubiquitination of a poly-ubiquitinated misfolded protein that was not degraded by the proteasome (192, 207). They are exposed to the cytosol and recruit the HDAC6 zinc-finger ubiquitin-binding domain (ZnF-UBP). HDAC6 binds to M1 and links the capsid to dynein-, actomyosin- and autophagy-dependent aggresome processing. The generated pulling force breaks the capsid, promotes vRNP release and infection (25, 191, 192) **(Figure 3)**. Unanchored poly-ubiquitin chains are emerging as key factors in multiple cellular responses, including innate antiviral pathways (208). It will be interesting to find out whether the incoming ubiquitin chains regulate downstream signaling events during and after influenza uncoating.

How does influenza virus prevent premature capsid uncoating during assembly? During the virus replication phase, HDAC6 undergoes caspase-mediated cleavage which inactivates both its ZnF-UBP and deacetylating enzyme (58, 209). This prevents premature capsid uncoating during virus assembly, and induces hyper-acetylated microtubules, which in turn, promote viral egress and budding (58, 210).

b) Ubiquitin proteasome system. Vaccinia virus core proteins are ubiquitinated during assembly and

packaged into virions. Viral core uncoating is driven by these pre-packaged ubiquitinated proteins, rather than by de novo ubiquitination. The core is primed for uncoating by the acidic pH of macropinosomes, most likely through a proton channel in the viral membrane (211). Following fusion, the core disulfide bonds are reduced in the cytosol. This and proteasome activity promote the disassembly of the lateral bodies (proteinaceous structures flanking the core of the virion), and the release of the viral phosphatase VH1 (212). VH1 dephosphorylates signal transducer and activator of transcription 1 (STAT1) and protects the infected cells from IFN restriction (212). In addition, core disassembly is dependent on the viral D5 primase/helicase, implicating that chemical energy is required for activating the process (145, 213-215).

c) ERAD/disaggregation. Polyomavirus is a non-enveloped virus that hijacks the ERAD machinery during entry. Following binding to GM1 gangliosides, SV40 arrives in the ER as largely unmodified particles (216-218). Uncoating is initiated in the ER, where the capsid diameter shrinks from 45 to 34nm (17, 219, 220). The capsid is remodeled structurally and exposes a hydrophobic peptide which is inserted into the ER membrane (16, 18, 183). The hydrophobic sequence recruits the ERAD, and the cytosolic protein disaggregation machinery Hsp105, and the small glutamine-rich tetratricopeptide repeat-containing protein α (SGTA)-Hsc70 complex, which together translocate the penetrating capsid into the cytosol (17, 19-23). Capsid uncoating, specifically the loss of interaction between the capsid protein VP1 and VP1 pentameric capsomer, may be aided by low Ca^{2+} concentrations in the cytosol (16, 221, 222). Subviral particles are imported into the nucleus via NLSs exposed during cytosolic uncoating (223-225).

d) Protease cleavage. Cleavage of the viral fusion protein is often crucial for entry, infection, and pathogenicity, since it enables the protein to receive cues from the host and insert into a host target membrane (24). For example, coronavirus (CoV) fusion (S) glycoprotein is primed by receptor binding and by low pH. It is proteolytically activated by endosomal cathepsins, and the cell surface transmembrane protease/serine (TMPRSS) proteases, furin, and trypsin (226, 227). These steps are spatiotemporally controlled. Binding of severe acute respiratory syndrome coronavirus (SARS-CoV) to its receptor angiotensin I converting enzyme 2 (ACE2) potentiates the S protein for cleavage by cathepsin L (228-231). The virus acquires fusogenicity in NPC1-positive endolysosomes where cathepsin L activity is high (232). Likewise, the fusion (F) protein of respiratory syncytial virus (RSV) undergoes cleavage by a furin-like protease twice (233, 234). The first cleavage happens during replication in producer cells. After macropinocytic uptake, a second cleavage in F provides the cue for penetration by an acid-independent membrane fusion mechanism (235). The human papillomavirus (HPV) capsid protein L1 is cleaved in the extracellular space by a serine protease Kallikrein 8. This cleavage is crucial for further conformational changes of the minor capsid protein L2 and optimal uncoating (148).

e) Intracellular lysosomal receptor. Ebola and Lassa virus use intracellular lysosomal receptors to penetrate into the cytosol. As shown in **Figure 4**, Ebola virus binds to T-cell immunoglobulin and mucin domain 1 (TIM-1) and AXL receptor tyrosine kinase for macropinocytic uptake by apoptotic mimicry (31, 236, 237). After successive proteolytic priming of the glycoprotein (GP) by cathepsin L and cathepsin B within acidic vesicles, GP and the virus-bound TIM-1 interact with the endolysosomal receptor NPC1, leading to viral fusion with the limiting endosomal membrane (9, 238-244). A monoclonal antibody against TIM-1 inhibited membrane fusion of several filoviruses (244). Fusion and endosomal escape of Ebola virus appears to happen from endolysosomes positive for both NPC1 and TPC2 (two pore Ca^{2+} channel 2) (232, 245, 246). TPC1 and 2 are major endosomal Ca^{2+} channels activated by nicotinic acid adenine dinucleotide phosphate (NAADP), and may influence endosome maturation by regulating release of Ca^{2+} from endolysosomes (247, 248).

Lassa virus, an arenavirus, binds O-glycans on cell surface dystroglycans for endocytic uptake (249). Low pH in late endosomes releases GP from dystroglycan and in turn promotes binding to N-glycosylated LAMP1. This receptor switching process activates membrane fusion and virus penetration (10, 250, 251).

f) Endosome maturation. The endosomal network that receives the incoming viruses is composed of several different types of organelles. These are involved in complicated trafficking, sorting, and maturation processes encompassing hundreds of cellular factors. During endosome maturation the incoming viruses can gain exclusive exposure to cues that are not available on the plasma membrane or in the cytosol (27, 28). This is supported by the notion that defects in endosome or macropinosome maturation on the pathway from the plasma membrane to lysosomes inhibits the productive entry of many viruses (8, 30, 202, 235, 252-263). Along the same lines, virus entry can be inhibited by perturbations of microtubule-mediated vesicular traffic, conversion of Rab5 early to Rab7 late endosomes, or the formation of intralumenal vesicles (27). In addition, endosomal cathepsins and furin-like proteases activate viral fusion proteins (264). Other viruses use endosomal membrane proteins as intracellular receptors to execute particular steps of their entry program (9, 10). Viruses that fail to escape from the endosome are eventually

degraded in lysosomes. Endosomes can also trigger innate immunity against viruses. In plasmacytoid dendritic cells, toll-like receptor 7 (TLR7) recognizes the ssRNA genomes contained within the influenza virion that are taken up into the endosome (265-267).

g) Co-opted lipid signaling. Non-enveloped viruses pierce or rupture the cell membrane in order to escape into the cytoplasm (268). As shown in **Figure 5**, adenovirus type 2 (HAdV-C2) uses membrane rupture twice, at the plasma membrane and endosomes, to gain entry into the cell. The virus first binds to its receptors coxsackie and adenovirus receptor (CAR) and integrins (269, 270). The combination of actomyosin-dependent drifting motion of CAR versus integrin-mediated confinement shears the capsid fibers and triggers conformational changes in the incoming capsid that enable externalization of the internal membrane lytic protein VI (11, 271). Protein VI contains an N-terminal amphipathic helix, and thereby creates small lesions in the plasma membrane, promoting cytosolic Ca^{2+} influx that in turn triggers lysosomal exocytosis and secretion of lysosomal acid sphingomyelinase (ASM) (272). ASM converts cell surface sphingomyelin to ceramide. This promotes virus uptake, which is also cholesterol- and dynamin-dependent (259, 260). Virus containing endosomes have a high ceramide level which favors the binding of protein VI to the lipid membrane and the disruption of the membrane. The concerted action of mechanical and chemical cues (actomyosin) together with receptors (CAR, integrin) and facilitators (ceramide) leads to enhanced rupture of the limiting membrane and escape of the virus from non-acidic endosomes to the cytosol (272).

Perspectives and challenges for the future

Virus entry and uncoating are distinct but highly interlinked processes (273). They are enabled by a wealth of pro-viral host factors (probably hundreds for each virus) and antagonized by host restriction factors that can preclude entry or trigger inappropriate virus disassembly (274). Both processes have classically been studied independent of each other. When the two were connected and analyzed with dedicated methodology, deep mechanistic insights have been obtained. We expect that novel cellular cues, facilitators, and viral uncoating strategies will be discovered in the future.

How will virus uncoating studies contribute to infectiology? An emerging challenge is to translate the results from cell culture experiments to primary cells and tissues, initially with the help of animal models and then human samples. Another challenge is to account for the fact that viruses are a cohort of particles infecting their target cell, tissue, organ, or organism. The entry pathways and uncoating factors may be different when cells are infected at low compared to high number of particles per cell (multiplicity). They may even depend on the nature of the producer and the target cells. For example, hepatitis A virus particles of the *picornaviridae* can be transmitted between cells as naked or lipid embedded capsids (275). The entry pathways for adenoviruses in epithelial cells are different from those in immune cells (276-278). In addition, the same virus can occur as diverse kinds of particles that use multiple entry pathways, such as spherical or filamentous influenza virus. All these features highlight the great adaptability and flexibility of natural infectious agents.

An emerging question for today's research in infectious disease is whether multiple virus infections affect one another. For this, methods to explore the complexity of the human virome in the host are being developed. For example, VirScan recently identified an increased rate of antibodies against adenovirus species C and RSV in HIV-positive human individuals compared with HIV-negative individuals (179). Are virus infections tuned by the bacterial microbiota? We believe that this is a relevant question, since mucosal surfaces of the oral, respiratory or intestinal tissues are major entry ports for viral pathogens into the human body, and these surfaces are colonized by microbiota, including billions of bacteria. We expect that in the near future the field of virus entry and uncoating will make increasing use of physiological model systems, quantitative omics, bioinformatics and eventually even personalized measurements.

Acknowledgements

This work was supported by VirX, a Swiss National Science Foundation (SNSF) SystemsX.ch medical research and development (MRD) grant, awarded to YY and UFG, and a grant from the SNSF (310030B_160316) to UFG. We thank S. Kilcher, A. Rudnicka, S. Stauffer, and M. Suomalainen for comments to the manuscript, and P. de Pablo for discussions. The authors declare no conflicts of interest.

Figure Legends

Figure 1. Endocytic pathways involved in virus entry. The majority of viruses use endocytosis for entry (4, 279). The virus-carrying vesicles and vacuoles often move along microtubules towards the nucleus. Cellular markers for the vesicles are shown within the light-blue boxes. The pH lowers as the vesicles mature and approach the nucleus. Viruses (not shown) enter the endocytic pathway and respond to cellular cues and facilitators that serve as uncoating signals. Such cues and facilitators are regulated in time and space and control the stepwise uncoating program, as shown in the examples in Figures 2-4. Abbreviatons: EEA1, early endosomal antigen 1; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes required for transport; LAMP1, lysosome associated membrane protein 1; NPC1, Niemann-Pick Disease, Type C1; PI3P, Phosphatidylinositol 3-phosphate; SNX, syntaxin; TGN, trans-Golgi network.

Figure 2. Cellular cues and processes implicated in viral uncoating. The scheme depicts cellular cues (receptors, enzymes, and chemicals including ions) and their cellular processes implicated in the stepwise uncoating of incoming virus particles. After binding to the cell surface via receptors and attachment factors, viruses are typically taken up into endocytic vacuoles following activation of signaling. They penetrate endosomes to enter the cytoplasm. Inside endosomes, viruses can be primed via endosome maturation, receptor binding, protease digestion, or ions. In some cases, viruses fuse directly at the plasma membrane to enter the cytoplasm. In the cytoplasm, viruses can be exposed to cellular cues and processes which culminate in the completion of uncoating and release of the viral genome from the capsid. The viral genome is transported to the site of replication, which may be in the cytosol, on cytoplasmic membranes or in the nucleus. Receptor cues (green), enzymatic cues (pink), chemical cues (blue), and cellular processes (white) implicated in viral uncoating are indicated. Facilitators are not shown.

Abbreviatons: ERAD, ER-associated protein degradation.

Figure 3. Cues and facilitators of Influenza A virus entry and uncoating. After binding to sialic acids on the cell surface, influenza A virus induces receptor tyrosine kinase (RTK) signaling via EGFR and endocytosis by CME or macropinocytosis (194, 197, 198, 280-282). CME involves Epsin1 and the virus particle enters Rab5-positive early endosomes (197). Macropinocytic uptake requires N-linked glycans on the cell surface, and also involves RTK signaling (194-196, 198). Endosome maturation and the influx of H^+/K^+ into the viral core via the M2 channel (shown by H^+/K^+ with white arrows) primes the virion for uncoating (199, 202, 203). Low pH in Rab7/LAMP1-positive late endosomes induces HA-mediated membrane fusion (as shown by H^+ with blue arrows) (206). Unanchored ubiquitin chains are exposed to the cytosol, followed by recruitment of HDAC6 and the aggresome processing machinery (including dynein, myosin, and the cytoskeleton) to disassemble the capsid shell by mechanical force (25). CD81 and cathepsin W also promote virus fusion (204, 205). Poly-ubiquitination of matrix protein M1 by E3 ubiquitin ligase Itch is also implicated in uncoating (283). Following capsid disassembly, the vRNPs are released into the cytosol, followed by NLS-mediated import into the nucleus, viral gene transcription and replication (110). Receptor cues (green), enzymatic cues (pink), chemical cues (blue), facilitators (brown), viral protein/process (grey), and endosomal markers (light blue) are indicated. The influenza virion scheme was adapted from visual-science.com.

Abbreviatons: EGFR, epidermal growth factor receptor; HA, hemagglutinin; LAMP, LAMP, lysosome associated membrane protein; uUb, unanchored ubiquitin; pUb, polyubiquitin; FACIL, facilitator; VIRAL, viral protein/process; MARK, endosomal marker; RECEP, receptor cue; ENZYM, enzymatic cue; CHEM, chemical cue.

Figure 4. Cues and facilitators of Ebola virus entry and uncoating. Ebola virus binds to the receptors TIM-1 and AXL and enters cells through classical apoptotic mimicry (31, 236, 237, 284). Endosome maturation, low pH, cathepsin L and cathepsin B activity prime the virus glycoprotein GP for fusion, an event that is also dependent on cellular factors PIKfyve and HOPS (250). Cathepsin activity is enhanced by low pH of endolysosomes (as shown by H^+ with arrows) (285). Once the virus reaches NPC1/TPC2-positive endosomes TIM-1 binds NPC1, which directly or indirectly activates fusion and penetration (244-246). TPC2 activity is regulated by NAADP, which is a highly potent intracellular calcium-mobilizing agent that stimulates intracellular calcium channels to release Ca^{2+} from endosomes and lysosomes, influencing the trafficking and maturation of endosomes (248, 286). Receptor cues (green), enzymatic cues (pink), chemical cues (blue), facilitators (brown), viral protein/process (grey), and endosomal markers (light blue) are indicated.

Abbreviatons: AXL, AXL receptor tyrosine kinase; GP, glycoprotein; HOPS, homotypic fusion and vacuole protein sorting; LAMP1, lysosome associated membrane protein 1; NAADP, nicotinic acid adenine dinucleotide phosphate; NPC1, Niemann-Pick Disease, Type C1; PIKfyve, FYVE finger-containing phosphoinositide kinase; PS, phosphatidylserine; TIM-1, T-cell immunoglobulin and mucin domain 1; TPC2, two pore Ca^{2+} channel 2; FACIL, facilitator; VIRAL, viral protein/process; MARK, endosomal marker, RECEP, receptor cue; ENZYM, enzymatic cue; CHEM, chemical cue.

Figure 5. Cues and facilitators of adenovirus entry and uncoating. HAdV-C2/C5 binds to CAR and integrins on the cell surface, and the actomyosin-dependent drifting motions of CAR trigger fiber shedding (2, 11, 12, 40). Virus binding to integrins induces signaling and virus uptake into endosomes in a dynamin-dependent manner (259, 260), Protein VI is dislocated from the inside of the virus and binds to the plasma membrane, forming small pores that allow influx of Ca^{2+} into the cytosol (272, 287). This danger signal induces rapid lysosomal secretion of ASM to the cell surface. ASM converts SM into CER, which enhances endocytic uptake of the virus. Protein VI is recruited to CER on the internal surface of endosomes, inducing endosomal leakage and rupture, and thereby enables escape of viral particles into the cytosol (260, 272, 288). Low pH is not required for virus penetration, but required to maintain functional secretory lysosomes (147). Receptor cues (green), enzymatic cues (pink), chemical cues (blue), facilitators (brown), viral protein/process (grey), and endosomal markers (light blue) are indicated.

Abbreviatons: ASM, acid sphingomyelinase; CAR, coxsackie and adenovirus receptor; CER, ceramide; ITGN, integrin; SM, sphingomyelin; FACIL, facilitator; VIRAL, viral protein/process; RECEP, receptor cue; ENZYM, enzymatic cue; CHEM, chemical cue.

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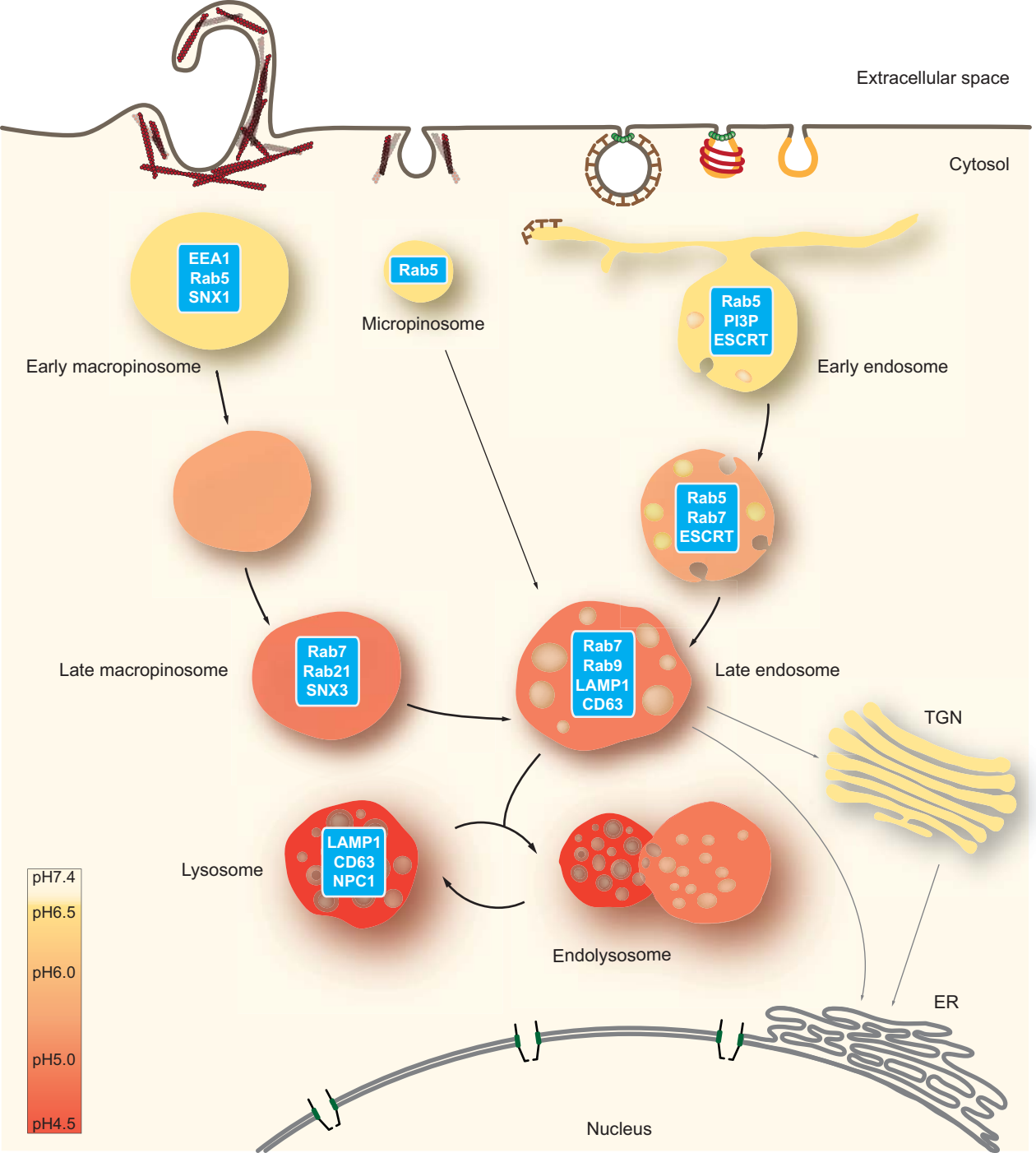
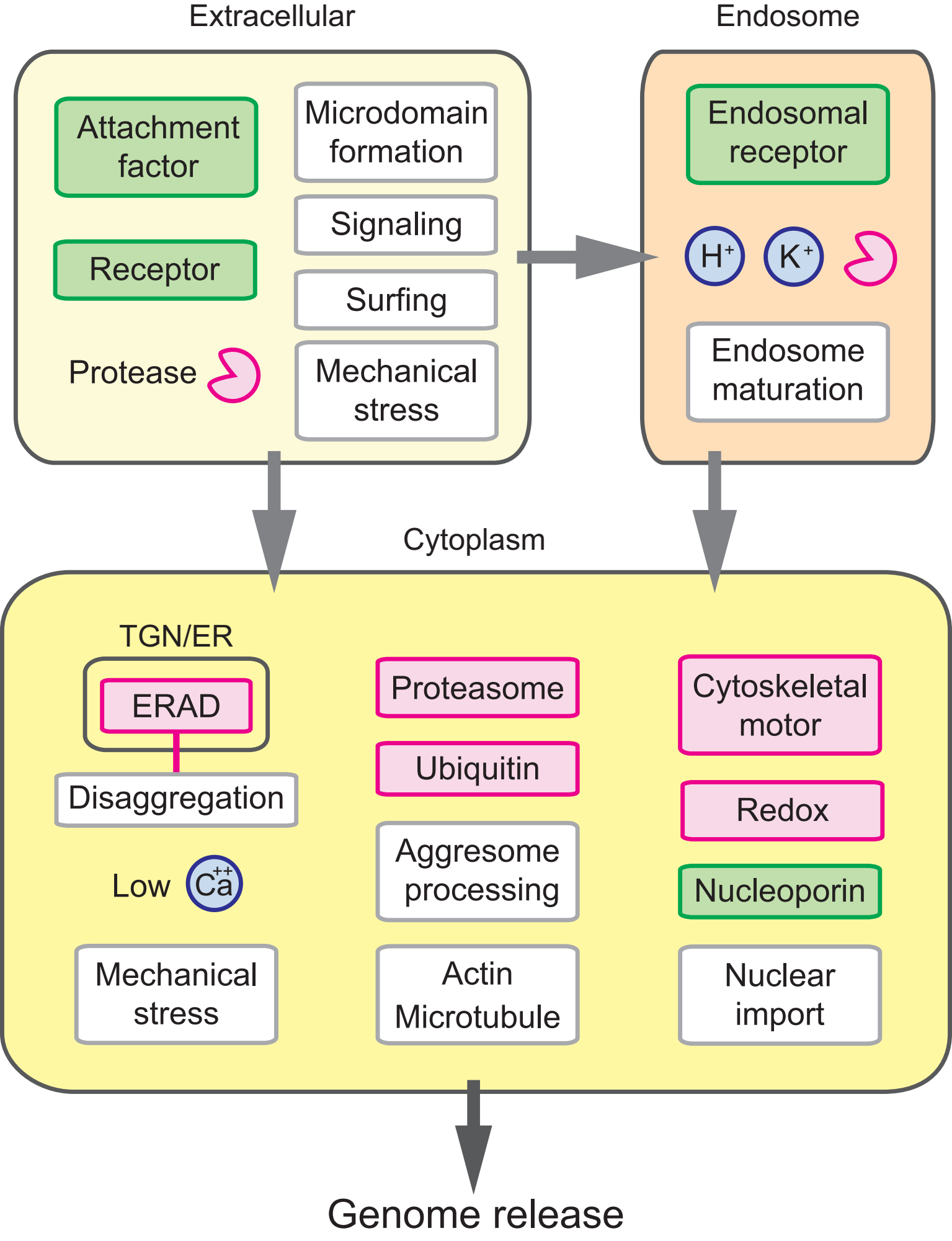
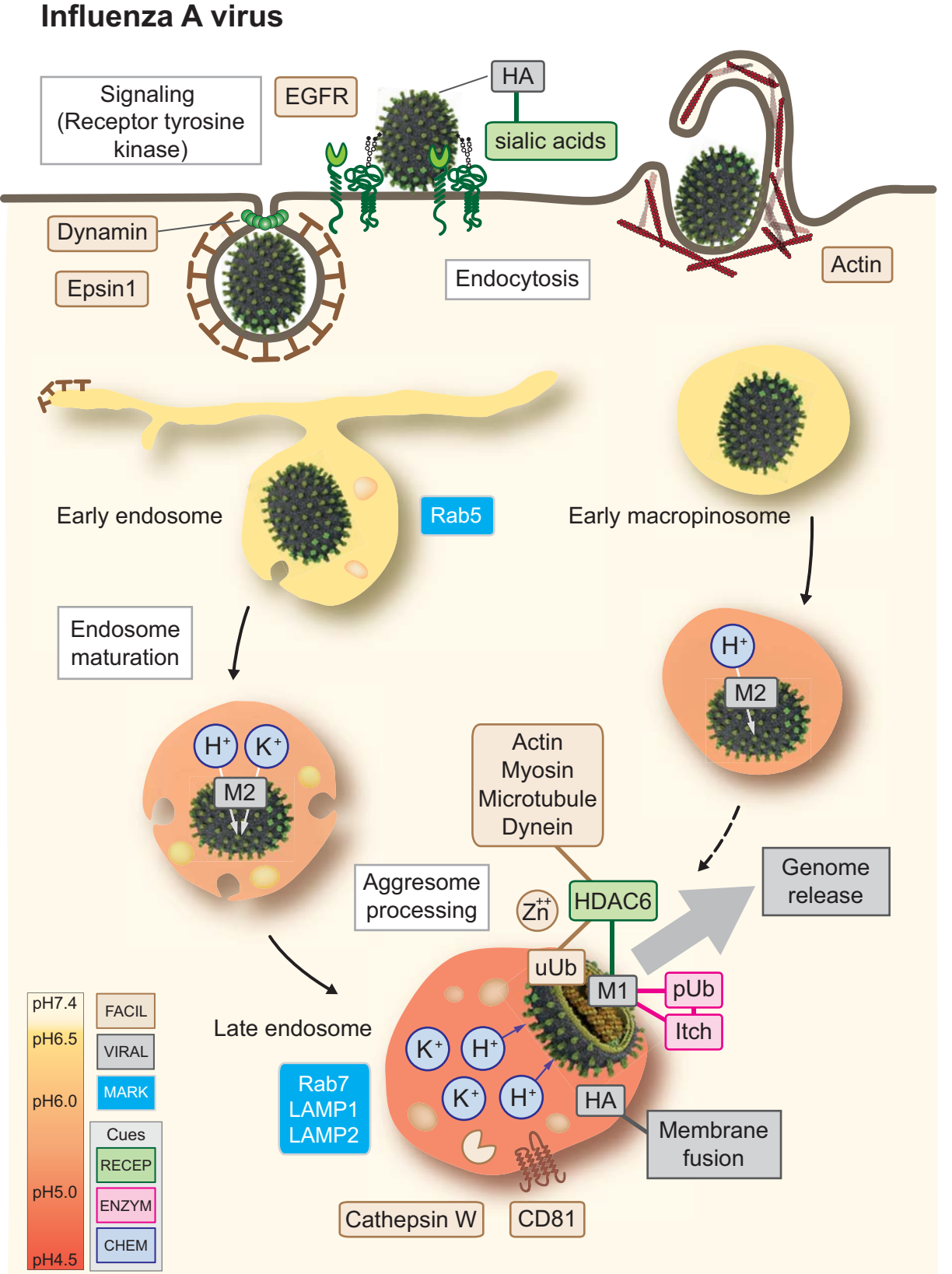
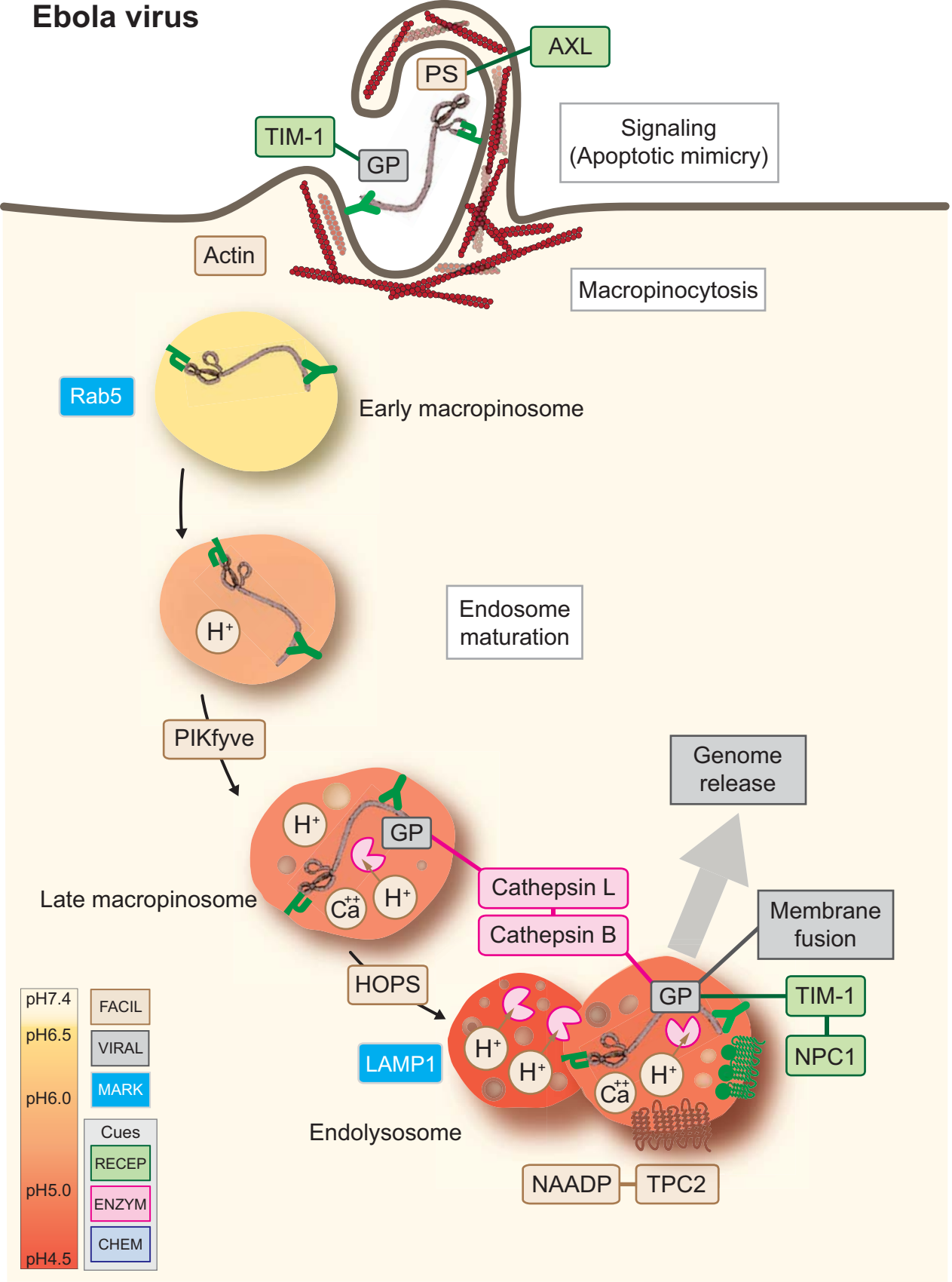

Extracellular space
Cytosol
EEA1
Rab5
SNX1
Early macropinosome
Rab5
Micropinosome
Rab5
PI3P
ESCRT
Early endosome
Rab5
Rab7
ESCRT
Rab7
Rab21
SNX3
Late macropinosome
Rab7
Rab9
LAMP1
CD63
Late endosome
TGN
LAMP1
CD63
NPC1
Lysosome
Endolysosome
ER
Nucleus
pH7.4
pH6.5
pH6.0
pH5.0
pH4.5


Extracellular
Endosome
Attachment factor
Receptor
Protease
Microdomain formation
Signaling
Surfing
Mechanical stress
Endosomal receptor
H+
K+
Endosome maturation
Cytoplasm
TGN/ER
ERAD
Disaggregation
Low Ca++
Mechanical stress
Proteasome
Ubiquitin
Aggresome processing
Actin Microtubule
Cytoskeletal motor
Redox
Nucleoporin
Nuclear import
Genome release


Influenza A virus
Signaling (Receptor tyrosine kinase)
EGFR
HA
sialic acids
Dynamin
Epsin1
Endocytosis
Actin
Early endosome
Rab5
Early macropinosome
Endosome maturation
H+
K+
M2
Actin
Myosin
Microtubule
Dynein
Aggresome processing
Zn++
HDAC6
Genome release
uUb
M1
pUb
Itch
Late endosome
Rab7
LAMP1
LAMP2
HA
Membrane fusion
Cathepsin W
CD81
pH7.4
pH6.5
pH6.0
pH5.0
pH4.5
FACIL
VIRAL
MARK
Cues
RECEP
ENZYM
CHEM


Ebola virus
AXL
PS
TIM-1
GP
Signaling
(Apoptotic mimicry)
Actin
Macropinocytosis
Rab5
Early macropinosome
H+
Endosome
maturation
PIKfyve
Genome
release
H+
GP
Ca++
H+
Late macropinosome
Cathepsin L
Cathepsin B
Membrane
fusion
HOPS
GP
TIM-1
NPC1
LAMP1
H+
H+
Ca++
H+
Endolysosome
NAADP
TPC2
pH7.4
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pH5.0
pH4.5
FACIL
VIRAL
MARK
Cues
RECEP
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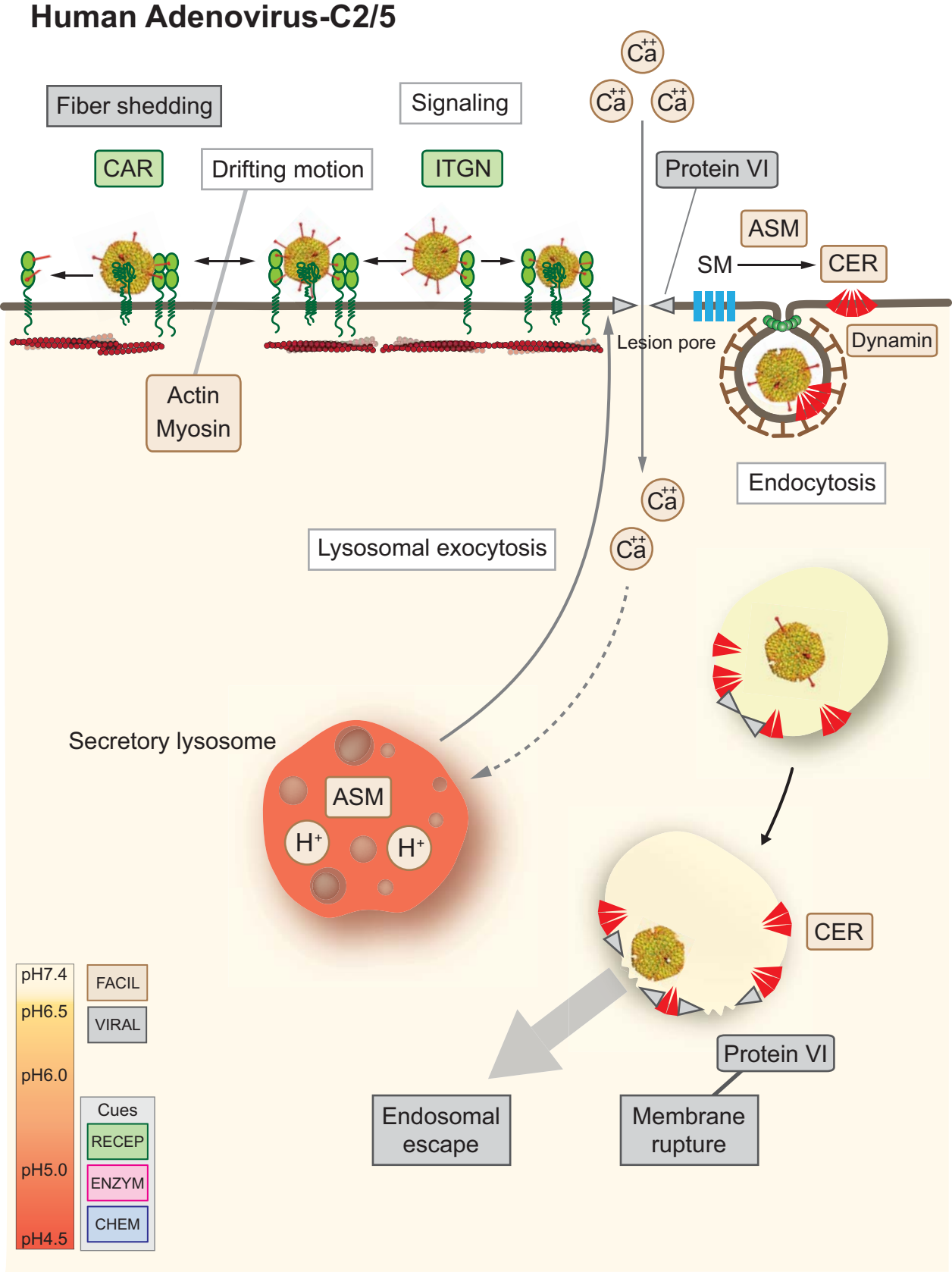
Human Adenovirus-C2/5
Fiber shedding
Signaling
Ca++
Ca++
Ca++
CAR
Drifting motion
ITGN
Protein VI
ASM
SM
CER
Lesion pore
Dynamin
Actin
Myosin
Endocytosis
Ca++
Ca++
Lysosomal exocytosis
Secretory lysosome
ASM
H+
H+
CER
Protein VI
Endosomal escape
Membrane rupture
pH7.4
pH6.5
pH6.0
pH5.0
pH4.5
FACIL
VIRAL
Cues
RECEP
ENZYM
CHEM